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# Isolation and characterization of *Pseudomonas syringae* pv. *porri* from leek in Flanders

**S. Rombouts · J. Van Vaerenbergh · A. Volckaert · S. Baeyen · T. De Langhe · B. Declercq · R. Lavigne · M. Maes**

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**Abstract** *Pseudomonas syringae* pv. *porri* causes bacterial leaf spot and blight of leek (*Allium porrum*) and is in wet crop seasons responsible for substantial losses. The local diversity within this pathogen in Flanders, Belgium, was investigated to obtain insights into its epidemiology. Therefore, symptomatic leek leaves were collected from 112 fields and bacteria were isolated. An oxidase negative, HR positive, fluorescent *Pseudomonas* was consistently recovered from the diseased tissues. Isolates were identified as *P. syringae* pv. *porri* by *rpoD* gene sequencing and by confirmation of pathogenicity in leek. Genomic profiles generated with BOX-PCR subdivided them into two groups, with one group containing 5 of the 37 analyzed strains. Those five isolates were all obtained in 2012 and the plant origins indicated seed transmitted infection. Draft genome sequences were produced for a *P. syringae* pv. *porri* strain from each BOX group and sequences of seven housekeeping genes were extracted for multi locus sequence analysis (MLSA). This resulted in the clustering of both *P. syringae* pv. *porri* strains with the *P. syringae* pv. *oryzae* strain 1_6 as did the whole genome sequence comparisons by ANI analysis. The *P. syringae* pv. *porri* isolates, designated LMG 28495 and LMG 28496, differed in a type III effector gene, *HrpW*, and in the number of mobile elements in the genome. Overall, the data demonstrate that two *P. syringae* pv. *porri* variants are present in symptomatic leek in Flanders which can be discriminated and possibly traced using a genomic profiling method such as BOX-PCR . Furthermore, the draft genome sequences of both strains will facilitate the development of sensitive and specific methods for early detection.

**Keywords** Bacteria · BOX-PCR · Genome analysis · Phylogenetics

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## Introduction

Leek bacterial blight was first reported as being caused by a *Pseudomonas* bacterium in 1952 by Lelliot and two decades later, in 1975, Hale attributed the pathogen to *P. syringae*. This bacteria was then classified as a new

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pathovar, *P. syringae* pv. *porri*, based on extensive research by Samson et al. in 1998. Leek (*Allium porrum*) is the major host, but the pathogen has also been diagnosed on onion (*Allium cepa*) and on shallot (*Allium cepa* var. *aggregatum*) (Noble et al. 2006). Currently, the disease has been reported in the United Kingdom (Lelliott 1952), France (Samson et al. 1981), New Zealand (Hale 1975), The Netherlands (Janse 1982), Italy (Varvaro 1983), the United States (Koike et al. 1999), Australia (Noble et al. 2006), Greece (Glynos and Alivizatos 2006), Japan (Goto 1972) and Korea (Myung et al. 2011, 2012). Typical symptoms include leaf curling and yellowing of the middle vein in young plants and water soaked spots on older leaves and flowering stems (Noble et al. 2006; Samson et al. 1998). The disease is known to be transmittable by seed (Ikene et al. 2003; Koike et al. 1999) but crop waste also plays a role in contaminating new leek plants in the field (Van Overbeek et al. 2010).

Recently, a serious increase in the prevalence of leek bacterial blight in Flanders, Belgium was reported, probably because a growing number of Flemish farmers buy leek transplants from plant nurseries. High plant densities in those nurseries, combined with plant manipulations such as irrigation and mowing, promotes dissemination of the pathogen (Koike et al. 1999). This study investigates the causal agent associated with leek blight epidemics in the past few years. First, bacteria were isolated from symptomatic leek leaves. Next, the identity of the isolates was revealed by using morphological and physiological tests and sequencing of the *rpoD* gene fragment, which is a useful phylogenetic marker for classification in *Pseudomonas syringae* (Parkinson et al. 2011). Pathogenicity was analyzed in leek leaves. BOX-PCR fingerprinting provided information on the diversity and possible common origin of the isolates that are present in the Flemish leek production. In a final step, draft genome sequence analysis and MLSA were performed to unravel genetic specificities of the *P. syringae* pv. *porri* isolates and to clarify phylogenetic relationships with other *P. syringae* pathovars.

## Materials and methods

### Bacterial isolation

After surface disinfection, small pieces at the margin of symptomatic leek leaf tissue were comminuted in 2 ml of sterile 10 mM phosphate buffer (PB) and decimal dilutions were spread onto Difco™ Pseudomonas Agar F (PAF, Becton Dickinson). Plates were incubated at 28 °C for 2–3 days and then checked for the presence of fluorescent colonies (Lelliot and Stead 1987). Grayish colonies with bluish fluorescence were consistently isolated from the extract dilutions and transferred to PAF to obtain a pure culture. The bacterial strains used in this study are listed Table 1. Thirty seven *P. syringae* pv. *porri* isolates were obtained in the frame of this study (GBBC numbers and the two representative isolates deposited as LMG 28495 and LMG 28496). The most recent isolates were obtained from symptomatic leek plants which were grown from transplants produced in nurseries in Belgium, the Netherlands and Morocco. Three cultures from a certified collection and one isolate from soil (Van Overbeek et al. 2010) were added as benchmark strains. Other strains included in the study were phylogenetically related to *P. syringae* pv. *porri* or were associated with diseased leek. Isolates were maintained in cryopreservation in Luria Broth supplemented with glycerol (20 % end concentration). Fresh cultures from cryo stocks were used in the various tests.

### Identification and characterization

Presumptive identification of the isolates from leek was performed with relevant tests from the LOPAT test scheme used for identification of fluorescent pseudomonads (Lelliot and Stead 1987). Levan production was evaluated on sucrose peptone agar (50 g/l sucrose, 5 g/l peptone, 0.5 g/l $K_2HPO_4$, 0.25 g/l $MgSO_4.7H_20$, 15 g/l agar) after incubation for 48 h at 28 °C. Cytochrome C oxidase activity was scored with oxidase test strips (Merck Millipore) according to the manufacturer's instructions. The hypersensitivity response was tested by infiltration of bacterial suspensions in 10 mM PB (about $10^8$ cells/ml) in tobacco (cv. Xanthi NN) leaves. Buffer infiltrations were used as negative controls and the *P. syringae* pv. *porri* pathovar reference strain CFBP $1908^{PT}$ served as positive control.

Further identification was done using antibodies raised against *P. syringae* pv. *porri* (IgG antiserum I-9534-01, Prime Diagnostics, Plant Research International, Wageningen, the Netherlands). Slide agglutination tests were performed with 24 h cultures on PAF from which separate colonies were suspended in 500 µl of 10 mM PB to give a turbid suspension of at least $10^9$ cells/ml. On a multispot slide, 50 µl of the bacterial suspensions were mixed with 10 µl of the fivefold diluted antiserum. The

**Table 1** Bacterial isolates and strains

| Strain number[a] | Plant origin | Geographical origin | Year of isolation | Identification |
|---|---|---|---|---|
| GBBC 715 | *Allium porrum* (leek) | Belgium | 2001 | *Pseudomonas syringae* pv. *porri* |
| GBBC 722 | *Allium porrum* (leek) | Belgium | 2001 | *Pseudomonas syringae* pv. *porri* |
| GBBC 728 | *Allium porrum* (leek) | Belgium | 2002 | *Pseudomonas syringae* pv. *porri* |
| GBBC 747 | *Allium porrum* (leek) | Belgium | 2002 | *Pseudomonas syringae* pv. *porri* |
| GBBC 1088 | *Allium porrum* (leek) | Morocco | 2011 | *Pseudomonas syringae* pv. *porri* |
| GBBC 1089 | *Allium porrum* (leek) | Morocco | 2011 | *Pseudomonas syringae* pv. *porri* |
| GBBC 1090 | *Allium porrum* (leek) | Morocco | 2011 | *Pseudomonas syringae* pv. *porri* |
| GBBC 1113 | *Allium porrum* (leek) | Belgium | 2003 | *Pseudomonas syringae* pv. *porri* |
| GBBC 1165 | *Allium porrum* (leek) | Belgium | 2004 | *Pseudomonas syringae* pv. *porri* |
| GBBC 1166 | *Allium porrum* (leek) | Belgium | 2004 | *Pseudomonas syringae* pv. *porri* |
| GBBC 1170 | *Allium porrum* (leek) | Belgium | 2004 | *Pseudomonas syringae* pv. *porri* |
| GBBC 1184 | *Allium porrum* (leek) | Belgium | 2004 | *Pseudomonas syringae* pv. *porri* |
| GBBC 1255 | *Allium porrum* (leek) | Belgium | 2005 | *Pseudomonas syringae* pv. *porri* |
| GBBC 1256 | *Allium porrum* (leek) | Belgium | 2005 | *Pseudomonas syringae* pv. *porri* |
| GBBC 1267 | *Allium porrum* (leek) | Belgium | 2005 | *Pseudomonas syringae* pv. *porri* |
| GBBC 1269 | *Allium porrum* (leek) | Belgium | 2005 | *Pseudomonas syringae* pv. *porri* |
| GBBC 1272 | *Allium porrum* (leek) | Belgium | 2005 | *Pseudomonas syringae* pv. *porri* |
| GBBC 1273 | *Allium porrum* (leek) | Belgium | 2005 | *Pseudomonas syringae* pv. *porri* |
| GBBC 1277 | *Allium porrum* (leek) | Belgium | 2006 | *Pseudomonas syringae* pv. *porri* |
| GBBC 1286 | *Allium porrum* (leek) | Belgium | 2006 | *Pseudomonas syringae* pv. *porri* |
| GBBC 1311 | *Allium porrum* (leek) | Belgium | 2007 | *Pseudomonas syringae* pv. *porri* |
| GBBC 1424 | *Allium porrum* (leek) | Belgium | 2012 | *Pseudomonas syringae* pv. *porri* |
| GBBC 1426 | *Allium porrum* (leek) | Belgium | 2012 | *Pseudomonas syringae* pv. *porri* |
| GBBC 1427 | *Allium porrum* (leek) | Belgium | 2012 | *Pseudomonas syringae* pv. *porri* |
| GBBC 1428 | *Allium porrum* (leek) | Belgium | 2012 | *Pseudomonas syringae* pv. *porri* |
| GBBC 1433 | *Allium porrum* (leek) | Belgium | 2012 | *Pseudomonas syringae* pv. *porri* |
| GBBC 1434 | *Allium porrum* (leek) | Belgium | 2012 | *Pseudomonas syringae* pv. *porri* |
| GBBC 1435 | *Allium porrum* (leek) | Belgium | 2012 | *Pseudomonas syringae* pv. *porri* |
| GBBC 1438 | *Allium porrum* (leek) | Belgium | 2012 | *Pseudomonas syringae* pv. *porri* |
| GBBC 1444 | *Allium porrum* (leek) | Belgium | 2012 | *Pseudomonas syringae* pv. *porri* |
| GBBC 1452 | *Allium porrum* (leek) | Belgium | 2012 | *Pseudomonas syringae* pv. *porri* |
| GBBC 1459 | *Allium porrum* (leek) | Belgium | 2013 | *Pseudomonas syringae* pv. *porri* |
| GBBC 1462 | *Allium porrum* (leek) | Belgium | 2013 | *Pseudomonas syringae* pv. *porri* |
| GBBC 1893 | *Allium porrum* (leek) | The Netherlands | 2013 | *Pseudomonas syringae* pv. *porri* |
| GBBC 1894 | *Allium porrum* (leek) | The Netherlands | 2013 | *Pseudomonas syringae* pv. *porri* |
| LMG 28495 | *Allium porrum* (leek) | Belgium | 2011 | *Pseudomonas syringae* pv. *porri* |
| LMG 28496 | *Allium porrum* (leek) | Belgium | 2012 | *Pseudomonas syringae* pv. *porri* |
| P55[b] | soil | The Netherlands | 2010 | *Pseudomonas syringae* pv. *porri* |
| CFBP 1908$^{PT}$ | *Allium porrum* (leek) | France | 1978 | *Pseudomonas syringae* pv. *porri* |
| CFBP 1687 | *Allium porrum* (leek) | United Kingdom | 1949 | *Pseudomonas syringae* pv. *porri* |
| CFBP 1770 | *Allium porrum* (leek) | New Zealand | 1973 | *Pseudomonas syringae* pv. *porri* |
| CFBP 3228$^{PT}$ | *Oryza sativa* | Japan | 1983 | *Pseudomonas syringae* pv. *oryzae* |
| CFBP 1634$^{PT}$ | *Coffea arrabica* | Brazil | 1958 | *Pseudomonas syringae* pv. *garcae* |
| CFBP 1674$^{PT}$ | *Avena sativa* | - | 1958 | *Pseudomonas syringae* pv. *striafaciens* |
| CFBP 2216$^{T}$ | *Avena sativa* | United Kingdom | 1958 | *Pseudomonas syringae* pv. *coronafaciens* |

**Table 1** (continued)

| Strain number[a] | Plant origin | Geographical origin | Year of isolation | Identification |
|---|---|---|---|---|
| CFBP 4117$^{PT}$ | *Zizania aquatica* | United States | 1983 | *Pseudomonas syringae* pv. *zizaniae* |
| CFBP 1617$^{PT}$ | *Beta vulgaris* | United States | 1959 | *Pseudomonas syringae* pv. *aptata* |
| LMG 1247$^{PT}$ | *Syringa vulgaris* | United Kingdom | 1950 | *Pseudomonas syringae* pv. *syringae* |
| LMG 1794$^{T}$ | pre-filter water-works tanks | United Kingdom | 1951 | *Pseudomonas fluorescens* |
| GBBC 1481 | *Allium porrum* (leek) | Belgium | 2011 | *Pseudomonas fluorescens* |
| GBBC 1480 | *Allium porrum* (leek) | Belgium | 2012 | *Pseudomonas fluorescens* |
| LMG 5100 | *Brassica oleracea* | United States | 1964 | *Pseudomonas viridiflava* |

[a] GBBC: culture collection of plant pathogenic bacteria at ILVO; CFBP: Collection Française de Bactéries Phytopathogènes, INRA Angers; LMG: Belgian Coordinated Collections of Microorganisms at the Laboratory of Microbiology of Ghent University with $^{T}$ as type strains and $^{PT}$ as pathovar reference strains

[b] received from Van Overbeek et al. 2010

slides were placed on damp paper tissue and agglutination was recorded within 2 min.

## PCR and sequencing

Bacterial isolates and strains were cultured for 24 h on PAF and DNA was extracted with the DNeasy Blood & Tissue kit (Qiagen) according to the instructions of the manufacturer. DNA concentration and purity was verified with the NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific Inc.). A fragment of the *rpoD* gene was PCR-amplified with the primers PsrpoD FNP1 (5′-TGAAGGCGARATCG AAATCGCCAA-3′) and PsrpoDnprpcr1 (5′-YGCMGWCAGCTTYTGCTGGCA-3′) (Parkinson et al. 2011). The PCR was performed in a 25 μl volume containing 1 μl DNA (25 ng/μl), 2.5 μl 10× PCR reaction buffer with 20 mM $MgCl_2$ (Roche), 2.5 μl dNTPs (2 mM each), 0.75 μl forward primer (10 μM), 1.5 μl reverse primer (10 μM) and 0.125 μl FastStart polymerase (5U/μl; Roche). The PCR temperature profile was according to Parkinson et al. (2011). For the amplification of the *HrpL* and *HrpS* gene, the protocol described by Sawada et al. (1999) was followed. All amplification products were purified with the GeneJET PCR purification kit (Thermo Fisher Scientific Inc.) and sequenced with both forward and reverse primers using a commercial service (Macrogen Inc.). The *rpoD* amplicons were trimmed to 578 nucleotides according to Parkinson et al. (2011) with Bionumerics 7.1 software (Applied Maths, Belgium). The 578 nt sequence was used to classify the bacterial isolates at the species level by a BlastN-query against the nucleotide database of NCBI. A phylogenetic tree was produced with the sequences of the bacterial isolates and reference strains using the UPGMA algorithm and bootstrap analysis of the Bionumerics software (Applied Maths, Belgium). The *hrpL* and *hrpS* sequences generated in this study are available at Genbank with accession numbers KT710765-KT710786.

## Pathogenicity tests

All fluorescent isolates from leek, along with reference strains from different *P. syringae* pathovars, were subjected to a pathogenicity assay on leek plants cv. Harston. Leek plants were cultured individually in pots in the greenhouse. Bacterial cultures were incubated on PAF plates for 24 h at 28 °C after which cell suspensions were prepared in 10 mM PB and adjusted to a concentration of about $10^7$ CFU/ml. For each isolate, 0.1 ml bacterial suspension was infiltrated with a syringe in two or three leaves of the same leek plant. Control leaves were inoculated with 10 mM PB. The inoculated plants were covered for 48 h to maintain humid conditions and they were kept at ambient temperature. The leaves were checked daily for symptom development with records after 7 and 12 days. The experiment was repeated once independently. Isolates or strains producing an atypical inoculation response were retested as described above using a cell suspension of about $10^5$ CFU/ml.

## BOX-PCR

BOX-PCR fingerprints were produced with primer BOXA1R developed by Martin et al. (1992). The PCR-protocol was adapted from Louws et al. (1994). It was

conducted in a 25 μl volume containing 4 μl DNA (25 ng/μl), 2.5 μl 10× PCR reaction buffer with 20 mM $MgCl_2$ (Roche), 2.5 μl dNTPs (2 mM), 2.5 μl BOXA1R primer (10 μM) and 0.2 μl FastStart polymerase (5 U/μl; Roche), and in a Biorad C1000$^{TM}$ Thermal Cycler using the following program: starting with 95 °C for 4 min; then 30 cycles of 95 °C for 30 s, 54 °C for 1 min and 68 °C for 8 min; and final extension at 68 °C for 8 min. The amplification products were separated by capillary gel electrophoresis on a QIAxcel Advanced Instrument (Qiagen) using a QIAxcel DNA High Resolution Gel Cartridge, 50 bp - 10 kb alignment markers and the OM1200 program of the QIAxcel Screengel 1.0.1.0. software. The profiles were imported using the Bionumerics Qiaxcel plug-in and analyzed with Bionumerics software.

### Genome sequence analysis

Strains LMG 28495 and LMG 28496 were used for whole genome analysis. DNA was prepared with the Qiagen Puregene kit and DNA quantity was verified with the Quantus fluorometer (Promega) using the Quantifluor DNA Quantitation kit (Promega). High molecular weight DNA integrity was verified on an 0.8 % agarose gel. Custom DNA library preparation and sequencing using multiplex Illumina Nextera technology was performed at BGI Tech Solutions, Hong Kong, China. A paired-end DNA library with 2×91 bp reads was constructed to generate assemblies with +/−100× coverage. Sequencing was performed on an Illumina Hiseq2000 instrument (Illumina Inc., San-Diego, USA). Initial quality assessment was based on data passing the Illumina Chastity filtering. Subsequently, samples were de-multiplexed and reads containing adapters, contamination or low-quality bases, were removed using an in-house filtering protocol from BGI. FastQ files were delivered by FTP. Quality trimming of the paired-end data set was performed in CLC Bio Genomics Workbench v7.0 (Aarhus, Denmark) using a quality score of 0.05 with a maximum of two ambiguous nucleotides per read. Then, *de novo* assembly was performed with the trimmed paired-end dataset with a minimum contig length of 200 bp using the EDENA v. 3.131028 and SOAPdenovo2-r240 ($k$=63) software for the genomes of LMG 28495 and LMG 28496 respectively (Hernandez et al. 2008; Luo et al. 2012). These draft genome scaffolds were subsequently ordered against the genome of *Pseudomonas syringae* pv. *tomato* DC3000 (RefSeq NC_004578) with Mauve V2.3.1 and concatenated using an in-house developed Perl script 'concatenate_genome.pl' with a six-frame stopcodon tag, 'CACACACTTAATTAATTAAGTGTGTG' between the contigs. Annotation of the draft genome was obtained with the online annotation tool RAST v4.0 (Aziz et al. 2008). The draft genome sequences of both strains are deposited at GenBank under accession numbers JTHM00000000 (LMG 28495) and JUEU00000000 (LMG 28496) after automatic annotation with the NCBI PGAAP online annotation pipeline (Angiuoli et al. 2008).

### Phylogenetic analysis

An *in silico* multi locus sequence analysis (MLSA) was performed using seven housekeeping genes (*gyrB*, *gapA*, *fruK*, *pgi*, *rpoD*, *acnB*, *gltA*) previously used by Sarkar and Guttman (2004), Hwang et al. (2005)and Baltrus et al. (2011). The gene fragments were extracted from the draft genomes of the two newly sequenced *P. syringae* pv. *porri* strains LMG 28495 and LMG 28496, and of the ones analyzed by Baltrus et al. 2011 (Table 3). Concatenated sequences of the seven genes were then aligned with ClustalW2 and a phylogenetic tree was constructed using MEGA6 software (Goujon et al. 2010; Tamura et al. 2013). The tree was calculated using the Maximum Likelihood method for calculating distances and the Neighbor Joining algorithm for clustering with 1000 bootstrap replicates. Furthermore ANIb (Average Nucleotide Identity) values were calculated for the same genomes using the python script 'calculate_ani.py' (Goris et al. 2007).

## Results

### Identification

A total of 112 blighted leek leaf samples were analyzed in this study. The majority was collected from leek growers in Flanders, Belgium, except for five transplant samples: two from the Netherlands and three from Morocco. In all these samples, we screened for colonies with a weak blue-white fluorescent appearance on PAF-medium under $UV_{366nm}$ irradiation and with a colony morphology similar to reference strains of *P. syringae* pv. *porri*. This led to the isolation of 37 presumptive *P. syringae* pv. *porri* isolates. No bacteria could be isolated from 28 leek samples and the rest of the samples contained bacteria with another colony morphology. Analysis of the *rpoD* or 16S rDNA sequence identified them as opportunistic soil bacteria such as

*P. fluorescens*. Two of the *P. fluorescens* isolates were retained for further characterization in this study. Our collection was supplemented with an isolate from the study of Van Overbeek et al. (2010), P55, and with reference strains from the CFBP and LMG collection (Table 1). Specific LOPAT tests were performed to discriminate *P. syringae* and *P. fluorescens* from other Pseudomonads. Oxidase production was tested to confirm the presence of cytochrome c as described by Samson et al. (1998), all suspected *P. syringae* isolates and *P. syringae* references were oxidase negative, in contrast with the suspected *P. fluorescens* isolates, which were indeed oxidase positive. To discriminate our isolates from *Pseudomonas viridiflava*, levan production was tested. All isolates produced levan in contrast to LMG 5100, our *P. viridiflava* reference strain. Production of the hypersensitivity reaction in the non-host Tobacco was also tested. With all the presumed *P. syringae* isolates from leek and with all other reference strains from *P. syringae*, the infiltrated leaf part developed necrosis. Also GBBC 1481 and GBBC 1480, both preliminary classified as *P. fluorescens*, reacted positive in Tobacco. The *P. fluorescens* reference LMG 1794$^{PT}$ did not induce a reaction after inoculation, possibly explained by its isolation source. To conclude, all presumed *P. syringae* isolates based on their blue-white fluorescence were oxidase negative, levan positive and produced a hypersensitive response, thus confirming their identity.

All 37 bluish fluorescent isolates displayed agglutination with the antiserum developed against *P. syringae* pv. *porri*. Likewise, the pathotype strain CFBP 1908$^{PT}$ and strains CFBP 1770, CFBP 1687 and P55 agglutinated. But in contradiction with the claims of the kit provider, the reaction was not specific, other related pathovar reference strains used in this study and the pathotype strain of *P. syringae* pv. *syringae* (Table 1) also showed agglutination There was no agglutination with the *P. fluorescens* isolates GBBC 1480, GBBC 1481 and LMG 1794$^{PT}$. This results demonstrate that slide agglutination is not appropriate for the identification of *P. syringae* pv. *porri* but it can be used as a fast screening tool to differentiate between *P. syringae* pv. *porri* and *P. fluorescens*.

## RpoD, HrpL and HrpS analysis

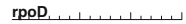
To confirm their presumed identity on a species level; all isolates with yellow or blue fluorescent colony morphology on PAF were analyzed by *rpoD* sequencing. BlastN comparison with GenBank reference sequences was used to identify genus and species type. The 37 isolates with weak blue-white fluorescence from leek were identified as *Pseudomonas syringae* and assigned to genomospecies 4 by the *rpoD* barcode, whereas all the yellow fluorescent isolates were not identified as *P. syringae*. *RpoD*-based barcoding was also completed for strain P55, the three reference strains of *P. syringae* pv. *porri* and the related pathovars *coronafaciens*, *oryzae*, *garcae*, *zizaniae* and *striafaciens* (all belonging to genomospecies 4 (Gardan et al. 1999)) and for the reference strains of *P. syringae* pv. *aptata*, *P. syringae* pv. *syringae* and *P. fluorescens*. All *P. syringae* pv. *porri* isolates had the same *rpoD* sequences and were also identical with that of CFBP 1674, the type strain of *P. syringae* pv. *striafaciens*. The other related pathovars had slightly different sequences. A phylogenetic tree was constructed with UPGMA analysis in Bionumerics (Fig. 1). Nucleotide sequences were translated with EMBOSS Sixpack, all *P. syringae* pathovars of genomospecies 4 investigated in this study had the same amino acid sequence within that *rpoD* fragment.

Because of the inability of the *rpoD* gene sequence to differentiate between the pathovars *porri* and *striafaciens*, the two pathogenicity-related genes *hrpL* and *hrpS* were sequenced for all *P. syringae* strains of this study. When considering the *hrpL* gene sequence, small differences were present between the sequence of *P. syringae* pv. *porri* (CFBP 1908$^{PT}$) and *P. syringae* pvs. *striafaciens* and *zizaniae* (CFBP 1674$^{PT}$ and CFBP 4117$^{PT}$). The other pathovars of genomospecies 4 had the same *hrpL* sequence as *P. syringae* pv. *porri*. Sequencing of the *hrpS* gene led to the discovery that only *P. syringae* pv. *porri* and *P. syringae* pv. *oryzae* (CFBP 3228$^{PT}$) had identic sequences, the sequence of the other pathovars differed slightly. These results demonstrate that *hrpL* and *hrpS* gene sequencing can be used in addition to the *rpoD* gene to differentiate the pathovars of genomospecies 4 on the sequence level.

## Pathogenicity tests

Pathogenicity was analyzed by inoculating leek plants. Typical symptoms of leaf blight were recorded for all

**Fig. 1** Phylogenetic tree produced by UPGMA-analysis with Bionumerics software showing the relationship between *P. syringae* pv. *porri* isolates and other related *P. syringae* pathovars based on a partial *rpo*D sequence. Similarity distances are given as percentage values and a cophenetic correlation coefficient was calculated with 1000 bootstrap replicates, values are shown above branches




| | | | | | | |
|---|---|---|---|---|---|---|
| CFBP 2216PT | Pseudomonas | syringae | coronafaciens | United Kingdom | 1958 |
| CFBP 3228PT | Pseudomonas | syringae | oryzae | Japan | 1983 |
| CFBP 1634PT | Pseudomonas | syringae | garcae | Brezil | 1958 |
| CFBP 4117PT | Pseudomonas | syringae | zizaniae | United States | 1983 |
| GBBC 1444 | Pseudomonas | syringae | porri | Belgium | 2012 |
| LMG 28496 | Pseudomonas | syringae | porri | Belgium | 2012 |
| CFBP 1674PT | Pseudomonas | syringae | striafaciens | | 1958 |
| CFBP 1687 | Pseudomonas | syringae | porri | United Kingdom | 1949 |
| CFBP 1770 | Pseudomonas | syringae | porri | New Zealand | 1973 |
| CFBP 1908PT | Pseudomonas | syringae | porri | France | 1978 |
| GBBC 1113 | Pseudomonas | syringae | porri | Belgium | 2003 |
| GBBC 1165 | Pseudomonas | syringae | porri | Belgium | 2004 |
| GBBC 1166 | Pseudomonas | syringae | porri | Belgium | 2004 |
| GBBC 1170 | Pseudomonas | syringae | porri | Belgium | 2004 |
| GBBC 1184 | Pseudomonas | syringae | porri | Belgium | 2004 |
| GBBC 1255 | Pseudomonas | syringae | porri | Belgium | 2005 |
| GBBC 1256 | Pseudomonas | syringae | porri | Belgium | 2005 |
| GBBC 1267 | Pseudomonas | syringae | porri | Belgium | 2005 |
| GBBC 1269 | Pseudomonas | syringae | porri | Belgium | 2005 |
| GBBC 1272 | Pseudomonas | syringae | porri | Belgium | 2005 |
| GBBC 1273 | Pseudomonas | syringae | porri | Belgium | 2005 |
| GBBC 1277 | Pseudomonas | syringae | porri | Belgium | 2006 |
| GBBC 1286 | Pseudomonas | syringae | porri | Belgium | 2006 |
| GBBC 1311 | Pseudomonas | syringae | porri | Belgium | 2007 |
| GBBC 1893 | Pseudomonas | syringae | porri | The Netherlands | 2013 |
| GBBC 1894 | Pseudomonas | syringae | porri | The Netherlands | 2013 |
| GBBC 715 | Pseudomonas | syringae | porri | Belgium | 2001 |
| GBBC 722 | Pseudomonas | syringae | porri | Belgium | 2001 |
| GBBC 728 | Pseudomonas | syringae | porri | Belgium | 2002 |
| GBBC 747 | Pseudomonas | syringae | porri | Belgium | 2002 |
| GBBC 1088 | Pseudomonas | syringae | porri | Morocco | 2011 |
| GBBC 1089 | Pseudomonas | syringae | porri | Morocco | 2011 |
| GBBC 1090 | Pseudomonas | syringae | porri | Morocco | 2011 |
| LMG 28495 | Pseudomonas | syringae | porri | Belgium | 2011 |
| GBBC 1424 | Pseudomonas | syringae | porri | Belgium | 2012 |
| GBBC 1426 | Pseudomonas | syringae | porri | Belgium | 2012 |
| GBBC 1427 | Pseudomonas | syringae | porri | Belgium | 2012 |
| GBBC 1428 | Pseudomonas | syringae | porri | Belgium | 2012 |
| GBBC 1433 | Pseudomonas | syringae | porri | Belgium | 2012 |
| GBBC 1434 | Pseudomonas | syringae | porri | Belgium | 2012 |
| GBBC 1435 | Pseudomonas | syringae | porri | Belgium | 2012 |
| GBBC 1438 | Pseudomonas | syringae | porri | Belgium | 2012 |
| P55 | Pseudomonas | syringae | porri | Netherlands | 2010 |
| GBBC 1452 | Pseudomonas | syringae | porri | Belgium | 2012 |
| GBBC 1459 | Pseudomonas | syringae | porri | Belgium | 2013 |
| GBBC 1462 | Pseudomonas | syringae | porri | Belgium | 2013 |
| LMG 1247PT | Pseudomonas | syringae | | United Kingdom | 1950 |
| CFBP 1617PT | Pseudomonas | syringae | aptata | United States | 1959 |
| GBBC 1480 | Pseudomonas | fluorescens | | Belgium | 2012 |
| GBBC 1481 | Pseudomonas | fluorescens | | Belgium | 2011 |
| LMG 1794T | Pseudomonas | fluorescens | | United Kingdom | 1951 |

presumed *P. syringae* pv. *porri* isolates and for the *P. syringae* pv. *porri* reference strains. The related pathovars, strains CFBP1617 (*Pseudomonas syringae* pv. *aptata*), CFBP1634 (*Pseudomonas syringae* pv. *garcae*), CFBP1674 (*Pseudomonas syringae* pv. *striafaciens*), CFBP3228 (*Pseudomonas syringae* pv. *oryzae*), CFBP4117 (*Pseudomonas syringae* pv. *zizaniae*) and CFBP2216 (*Pseudomonas syringae* pv. *coronafaciens*) as well as the *P. fluorescens* isolates GBBC 1480, GBBC 1481 and LMG 1794 did not induce blight symptoms in the leek leaves. The 37 presumed *P. syringae* pv. *porri* isolates were able to multiply and spread in the leaves and cause the typical symptoms of leek leaf blight beyond the place of inoculation. *P. syringae* pathovars *aptata*, *garcae*, *striafaciens* and *oryzae* induced a local reaction, displaying sunken necrotic lesions at and around the inoculation point. However, these strains did not produce any symptom in the leek leaves when infiltrated with the 1/100 diluted inoculum whereas greasy leaf spots and stripes were readily formed by *P. syringae* pv. *porri*.

## BOX-PCR

BOX-PCR fingerprinting is a tool that can be used to identify bacterial isolates at the genomospecies level (Marques et al. 2008; Rademaker and De Bruijn 1997) or to study genomic diversity (F. Louws et al. 1999). Furthermore it has already been used to determine the genetic diversity of *Pseudomonas* midrib rot isolates from lettuce and demonstrated a single inoculum source per greenhouse (Cottyn et al. 2009). To gain further insight into the genomic diversity between the *P. syringae* pv. *porri* isolates studied, genomic fingerprints were produced for all isolates. The *P. syringae* pv. *porri* isolates all showed a considerable degree of homogeneity in their BOX-fingerprint (Fig. 2), but a minor difference divided them into two groups. Isolates GBBC 1427, GBBC 1428, GBBC 1438, GBBC 1444 and LMG 28496 all display the same small difference discriminating them from the other *P. syringae* pv. *porri* isolates, indicating that the *P. syringae* pv. *porri* isolates do not constitute one homogenous group. Analyzing the sample information of those isolates revealed a common seed origin for four out of five isolates indicating the possibility of seed transmission. The related *P. syringae* pathovars from genomospecies 4 (CFBP 3228, CFBP 1634, CFBP 1674, CFBP 2216 and CFBP 4117) used in this study, showed a banding pattern very similar to the *P. syringae* pv. *porri* isolates.

## Genome sequence analysis

From each of both fingerprint groups a representative isolate was deposited at GenBank (LMG 28495 and LMG LMG 28496) and its draft genome assembled (Table 2). The higher amount of contigs obtained for LMG 28496 indicated that its draft genome quality was slightly less good than that of LMG 28495, but other metrics were comparable. After ordering the contigs against the genome of *P. syringae* pv. *tomato* DC3000 and concatenating them with a linker sequence they were compared with the RAST v4.0 tool (Aziz et al. 2008). According to RAST, differences between both strains were mostly situated in their prophage content and this result was confirmed with PHAST (PHAge Search Tool) (Zhou et al. 2011). The draft genome of LMG 28495 contained three intact and two incomplete prophages, while LMG 28496 had six intact and eight incomplete prophages throughout the genome. None of those prophage genes related to virulence or toxicity. Also other mobile elements such as IS elements varied. Using ISsaga (IS Semi-Automatic Genomic Annotation) (Varani et al. 2011), 51 IS elements were identified in the draft genome of LMG 28495 of which 29 different ISs, in contrast to 47 different ISs in LMG 28496 with a total of 103 ISs. When comparing subsystems in RAST, both strains reveal minor differences. LMG 28496, for example, encodes a type III effector translocator, *Hrp*W, which is not present in LMG 28495. This *Hrp*W gene is flanked by two mobile elements in LMG28496. Both LMG 28496 and LMG 28495 genomes contain *Hrp*Z1 and *Hrp*K1 homologs, which are also regarded as type III effector translocators.

## Phylogenetic analysis

To investigate the position of *P. syringae* pv. *porri* within the *P. syringae* species complex, an *in silico* MLSA was performed using the concatenated sequences of seven genes (*gyrB*, *gapA*, *fruK*, *pgi*, *rpoD*, *acnB*, *gltA*) (D. A. Baltrus et al. 2011; Hwang et al. 2005; Sarkar and Guttman 2004) from LMG 28495, LMG 28496 and 19 other *P. syringae* strains as available from their draft genomes in NCBI (Table 3). Our two *P. syringae* pv. *porri* strains clustered together with *P. syringae* pv. *oryzae* strain 1_6, which confirmed the internal relatedness shown with the *rpoD* barcode sequence (Fig. 3). MLSA research has previously been

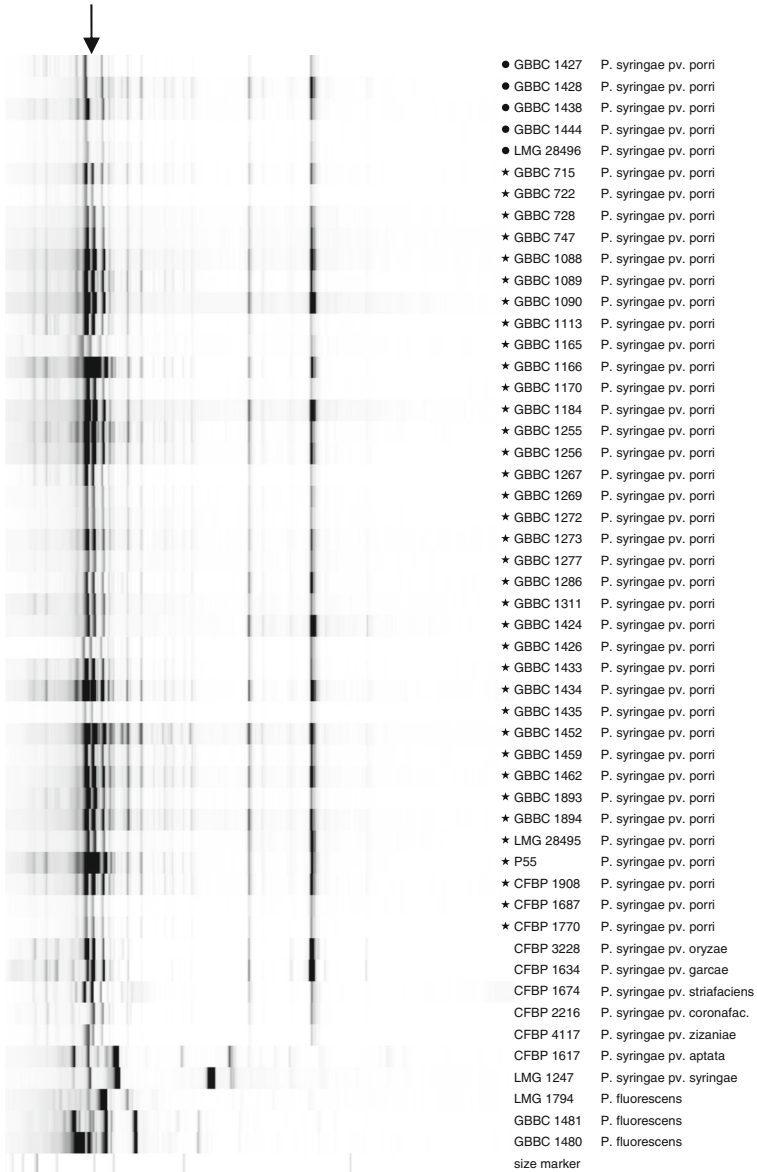



**Fig. 2** BOX-PCR patterns of all *P. syringae* pv. *porri* isolates and reference strains. The two symbols (* and °) indicate the two groups observed in BOX-PCR pattern and the arrow gives the position of the discriminative band. The QIAxcel® Size Marker 250 bp – 8 kb is added as a reference

used to divide strains of *P. syringae* pv. *actinidiae* into four groups based on the same housekeeping genes (Chapman et al. 2012). However, the *P. syringae* pv. *porri* strains we analyzed had identic sequences for the seven genes indicating that MLSA based grouping cannot be used to differentiate between them. As there is a possibility of incongruence between MLSA and whole-genome based phylogenies (Baltrus et al. 2013), a phylogenetic tree was constructed based on the ANIb values of the same *P. syringae* genomes (Fig. 4). Even though ANIb values are calculated by comparing complete genomes, they provided the same groups and relatedness as produced by MLSA. The calculated average nucleotide identity value of the two *P. syringae* pv. *porri* isolates LMG 28495 and LMG 28496 was 99.52 %.

## Discussion

The increase of bacterial blight of leek and the related economical losses attracted our interest in the problem a few years ago leading to an extensive investigation of the causative agent. All 37 blue fluorescent isolates from

**Table 2** Draft genome metrics of *P. syringae* pv. *porri* strains LMG 28495 and LMG 28496

| | LMG 28495 | LMG 28496 |
|---|---|---|
| genome size (bp) | 6 050 000 | 6 269 274 |
| GC% | 57.5 | 57.4 |
| contigs≥200 bp | 181 | 308 |
| N50 contig size nbp) | 96 630 | 102 657 |
| average coverage | 94 | 105 |

leek incorporated in this study were identified as *P. syringae* pv. *porri*. This was supported by their reaction in the selected LOPAT tests and their pathogenicity on leek. Evidence for their classification within *P. syringae* was further based on the *rpoD* barcode sequence and on comparison of the genomes of two of our isolates with available *Pseudomonas* sequences in NCBI with MLSA and ANIb. These taxonomic methods enable a robust demarcation of bacterial species (Marcelletti and Scortichini 2014). Remarkably, within this complex *P. syringae* species MLSA and ANIb displayed enough resolution power to show groupings at a sub-species level, and clearly clustered our two *P. syringae* pv. *porri* isolates with *P. syringae* pv. *oryzae*. In general, both methods divided the *P. syringae* strains in the same groups and the obtained groups were consistent with the clustering based on the *rpoD* barcode sequence alone as presented by Parkinson et al. in 2011. The fact that *P. syringae* pv. *porri* clusters together with the rice pathogen *P. syringae* pv. *oryzae* is maybe not completely irrelevant since both are pathovars of a monocot plant.

**Table 3** Strains used in MLST analysis

| Pathovar | Strain | NCBI Accession |
|---|---|---|
| *actinidiae* | MAFF302091 | AEAL |
| *aceris* | MAFF302273PT | AEAO |
| *aesculi* | 0893-23 | AEAD |
| *aptata* | DSM50252 | AEAN |
| *glycinea* | A29-2 | ADWY |
| *japonica* | MAFF301072PT | AEAH |
| *lachrymans* | MAFF301315 | AEAF |
| *lachrymans* | MAFF302278PT | AEAM |
| *maculicola* | ES4326 | AEAK |
| *mori* | MAFF301020 | AEAG |
| *morsprunorum* | MAFF302280PT | AEAE |
| *oryzae* | 1_6 | GCA_000156995.1 |
| *phaseolicola* | 1448a | GCA_000012205.1 |
| *pisi* | 1704B | AEAI |
| *syringae* | B728a | GCA_000012245.1 |
| *tabaci* | ATCC11528 | AEAP |
| *tomato* | DC3000 | GCA_000007805.1 |
| *tomato* | T1 | GCA_000172895.1 |
| *NA* | Cit7 | AEAJ |

The *Pseudomonas syringae* species complex to which this pathovar belongs consists of 57 different pathovars, many of them being plant pathogens infecting a wide range of hosts (Marcelletti and Scortichini 2014). Core genome analysis revealed that *P. syringae* is a highly clonal and stable species that is endemic within plant populations (Sarkar and Guttman 2004). Genetic variations outside the core genome, especially variations in type III effector repertoires, are responsible for the variation in plant hosts of the different pathovars (Baltrus et al. 2011). Within the pathovars small genomic variation is present that can be used to determine the origin of an epidemic, the spread of clonal lineages, the impact and effectiveness of control measures and the suitability of current pathogen detection protocols (Chevillon et al. 2012). In a related pathovar, *P. syringae* pv. *actinidiae*, differences in integrative conjugative elements (ICE) were noted between strains isolated from different geographic areas (Butler et al. 2013) but strains of the current outbreak had all the same Rep-PCR profiles (Ferrante et al. 2015). Because of the small geographic area covered by the strains of our study, one would expect them to form a homogenous group. In contrast, two genomic groups could be discriminated in the *P. syringae* pv. *porri* strain set of this study based on BOX-PCR profiling. The smallest group only contained five of the 37 isolates. These five were isolated in 2012 from three different leek fields in Flanders and from three different cultivars. Four of these isolates were obtained from plants produced from seeds that were retrieved from the same seed company, indicating the possibility they had been introduced through infected seeds. Earlier research already suggested spread of *P. syringae* pv. *porri* with contaminated seeds (Koike et al. 1999; Noble et al. 2006). All reference strains, some of them used in previous studies, are situated in the large BOX profile group suggesting that the five isolates from the small BOX group in 2012 constitute a new genotype. Genomic differences among *P. syringae* pv. *porri* strains were also described by Noble et al.

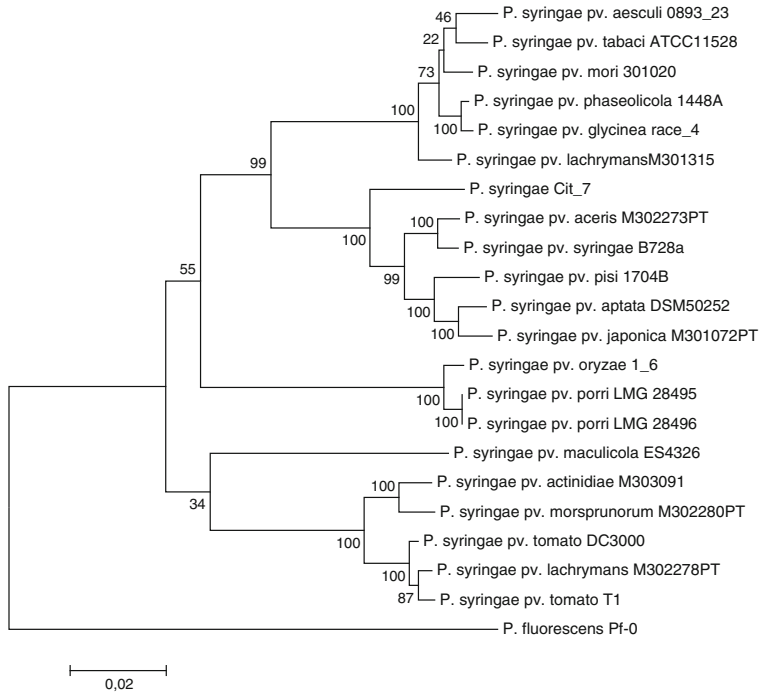



**Fig. 3** Phylogenetic tree of 21 *P. syringae* strains based on the concatenated sequences of seven conserved loci. The scale bar indicates similarity distances given as percentage values and a cophenetic correlation coefficient was calculated with 1000 bootstrap replicates

(2006), who showed minor differences based on profiles generated with IS50-primer amplification and RFLP of a 16S rDNA fragment. In contrast to our data, these authors as well as Koike et al. (1999) and Van Overbeek et al. (2010) did not discriminate different genotypes among their isolates from California, Australia and the Netherlands when applying different repetitive-element-PCRs (BOX, REP and ERIC).

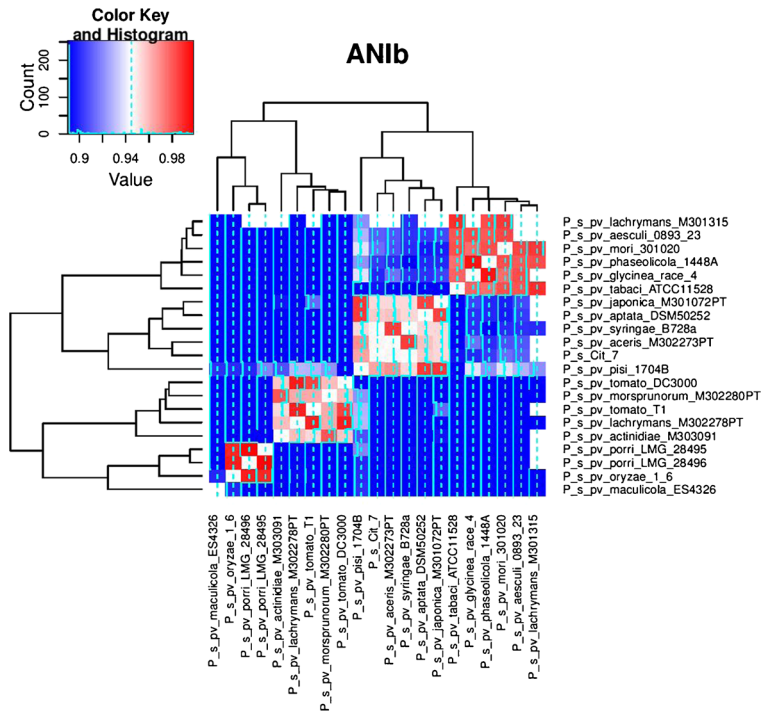



**Fig. 4** Phylogenetic tree demonstrating the relatedness of 21 *P. syringae* strains based on ANIb values of their genomes

Further analysis of the genomes of two representatives of both genotypes, LMG 28495 and LMG 28496, showed high similarity between both, but there were also some differences observed. Mobile elements such as prophages and IS elements were significantly more present in LMG 28496 than in LMG 28495. These mobile elements are known to be involved in genome rearrangements and strain differentiation in other plant pathogens such as *Xanthomonas* and *Xylella* (Varani et al. 2013). In addition, an *HrpW* gene bordered by mobile elements was detected in LMG 28496 and absent in LMG 28495. HrpW has been identified as a type III effector translocation protein in *P. syringae* pv. *tomato* DC3000 (Jin et al. 2001) and *P. cichorii* (Kajihara et al. 2012) and is associated with virulence and host range (O'Brien et al. 2011). In *P. syringae* pv. *tomato* DC3000, three other harpins are present: HrpZ1, HrpK1 and HopAK1, forming a consortium of semi-redundant translocators (Collmer et al. 2000; Kvitko et al. 2007). Both LMG 28495 and LMG 28496 contain *Hrp*Z1 and *Hrp*K1 homologs. Based on the leek inoculation tests that were performed we did not observe differences in pathogenicity between LMG 28495 and LMG 28496, which leaves uncertainty on the consequences for virulence or host range of the acquisition of *HrpW* by LMG 28496. Anyway, LMG 28496 belongs to the 5 isolates constituting the small Box-group and appears to have been submitted to several events of lateral gene transfer.

The data presented here confirms *P. syringae* pv. *porri* as the causal agent of leek bacterial blight, and seed as a possible pathway for spread. We also identified a genetically different *P. syringae* pv. *porri* type from the Flemish leek production fields of 2012. The genome analysis suggests that lateral gene transfer in this type could have been responsible for the acquisition of a gene associated with pathogenicity. The consequences for the fitness, virulence, host range and further spread of this bacterial type are difficult to predict, but the BOX-PCR and based on it a type-specific PCR that could be developed are appropriate tools to monitor its occurrence and further evolution.

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**Compliance with ethical standards**

**Conflict of interest** There are no conflicts of interest

**Human and Animal Rights and Informed Consent** No participation of humans or animals in the reported research.

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